

# Linking biofilm spatial structure to real-time microscopic oxygen decay imaging

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## **Abstract**

We combined two non-destructive techniques, confocal laser scanning microscopy (CLSM) and planar optode (ViviSens imaging), to relate fine-scale spatial structure of biofilm components to real-time images of oxygen decay in aquatic biofilms. To this aim, both techniques were applied to biofilms grown for 7 days at contrasting conditions of light and temperature (10/20°C). The geostatistical analyses of the CLSM images indicated that biofilm structures consisted of a combination of small size ( $\sim 10^0$   $\mu\text{m}$ ) and middle size ( $\sim 10^1$   $\mu\text{m}$ ) irregular aggregates. Cyanobacteria and EPS (extracellular polymeric substances) showed larger aggregate in 20°C dark-grown biofilms while, for algae, aggregates were larger in light-20°C conditions. Light-20°C biofilms were the densest ones, while dark-10°C

biofilms showed the sparsest structure and lowest respiration rates. There was a positive relationship between number of pixels occupied by biofilm and oxygen concentration decay rate. The combination of optodes and CLMS, taking advantage of a geostatistical analysis, is a promising way to relate biofilm architecture and metabolism at the micrometric scale.

**Keywords:** confocal laser scanning microscopy, real-time images of oxygen concentration, biofilm growth, planar optodes, biofilm respiration, geostatistics, nested structures

## 1. Introduction

Biofilms grow on wet surfaces and consist of a combination of autotrophic and heterotrophic microorganisms embedded in self-produced extracellular polymeric substances (EPS) (Costerton et al., 1978). They play a beneficial and critical role in the metabolism of water bodies, being responsible for most of the nutrient cycling, uptake and

mineralization processes occurring in aquatic ecosystems, as well as in natural and manmade infiltration devices (Romani et al., 2004; Gette-Bouvarot et al., 2014; Battin et al., 2016). Conversely, biofouling processes (Sala et al., 2013) represent the main adverse effect of biofilms in industrial water systems, including drinking water distribution systems, water treatment applications (such as porous membranes, Nguyen et al., 2012), and marine systems (e.g., aquaculture nets, oil and gas installations, and ship hulls). Both positive and negative macroscopic effects represent the collective result of processes occurring at microbial hotspot scales ( $\mu\text{m}$  to  $\text{cm}$ ). Analyzing the fine spatial-scale changes in biofilm structure and metabolism under different environmental conditions is of paramount

importance to better understanding the macroscopic effects of biofilm in the environment where it develops.

Biofilms present complex and dynamic heterogeneous structures, with sparse and dense zones modulated spatially and temporally by environmental drivers, such as nutrient availability, light, and flow rate (Singer et al., 2010; Proia et al., 2012; Mora-Gómez et al., 2016). Light influences the biofilm thickness, as well as the distribution and relative proportion of autotrophic and heterotrophic microorganisms, responsible for changes in respiration rates (Ylla et al., 2009). Directional flow, shear stress and soil heterogeneity also induce changes in the direction of biofilm growth, leading to anisotropic spatial distributions (e.g., Jung-Woo et al., 2010; Hödl et al., 2011; Bozorg et al., 2012) and the formation of preferential flow paths (Rubol et al. 2014). Another key environmental factor is temperature, which promotes fast biofilm colonization and enhances young biofilm growth (Díaz Villanueva et al., 2011). However, limited information is currently available

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on whether oxygen concentration distribution in space and time is affected by these environmental conditions and how it is linked to the biofilm architecture (Fenchel and Finlay, 2008).

A plethora of techniques are currently available to investigate the spatial distribution of microbial assemblages. These include, among others, confocal laser scanning microscopy (CLSM) and planar optodes. The former is a well established technique to study biofilm structure (New and Lawrence, 2016), as it provides simultaneous information about the three-dimensional structure of thin (young) biofilms (Barranguet et al., 2004) and the identification of different components by both auto-fluorescence (for algae and cyanobacteria) and fluorescent dyes (for DNA and EPS). The latter offers bidimensional

oxygen concentration images of surface biofilm based on dynamical quenching of a luminescence indicator by oxygen ( $O_2$ ). Optodes have been successfully applied in the study of microbial active soils and sediments (Rubol et al., 2016) and biofilms (Borzog et al, 2012; Staal et al, 2011a), with particular focus on the biofilm functioning (Kühl et al, 2007; Kühl and Polerecky, 2008). Both CLSM and optodes are non-destructive techniques capable to highlight differences in spatial distribution that cannot be properly observed by other point measurement devices such as optical fibers or micrometers.

The link between structure and metabolic function of biofilm is under current debate. Staal et al. (2011b) showed that biofilm in tap-water present distinct hotspots of activity modulated by flow rates, with no clear link to structure. Other works, however, suggest that biofilm structure and functionality are connected, but only limited information is available at the very fine scale (e.g., Proia et al, 2012).

Here, we quantify the link between the spatio-temporal concentration distribution of oxygen in the biofilm and the spatial structure of its components (algae, cyanobacteria,

bacteria, and EPS) with emphasis on the quantification of aggregate sizes. We used one-week old biofilms grown at four combinations of light and temperature conditions, processed by both CLSM and Visisens planar optode (PreSens Precision Sensing, Germany). The corresponding CLSM 3D and 2D information obtained were then used to determine the spatial statistics of biofilm growth. In parallel, the Visisens plus imaging technology allowed mapping the percentage of air saturation in the biofilm as a function of time and space. The combination of the two high precision techniques allowed studying the correlation of biofilm structure and oxygen consumption for some specific temperature and light conditions.

88

## 89 2. Materials and Methods

### 90 2.1 Biofilm growth

91 Artificial glass tiles (of area  $1\text{cm}^2$ ) were used as substrata for biofilm growth and were  
92 incubated in 8 microcosms with a biofilm extract inoculum obtained from an oligotrophic  
93 stream (Fuirosos stream) following the protocol outlined in Ylla et al. (2009). Each  
94 microcosm consisted of a sterile glass jar (19 cm in diameter, 9 cm high) with autoclaved  
95 glass tiles attached to the bottom. Microcosms were filled with 1.5 L of water of known  
96 chemical composition mimicking that of the Fuirosos stream, and obtained by dissolving  
97 some pure salts (12 mg/L  $\text{Na}_2\text{SO}_4$ , 20 mg/L  $\text{Na}_2\text{SiO}_3$ , 30 mg/L  $\text{CaCl}_2$ , 1 mg/L  $\text{KCl}$ , 2 mg/L  
98  $\text{MgSO}_4$ , and 20 mg/L  $\text{NaHCO}_3$ ) in MilliQ water. Water was continuously recirculated by  
99 means of a submersible pump (Hydor, Pico 300, 230V, 50 Hz, 4.5 W) (one pump for each  
100 microcosm).

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101 The microcosms were placed inside an incubator (SCLAB-PGA500) at four  
102 different combinations of light and temperature (with two replicates in each case): Light-  
103  $10^\circ\text{C}$  (from now on denoted as 10L), Dark- $10^\circ\text{C}$  (10D), Light- $20^\circ\text{C}$  (20L), and Dark- $20^\circ\text{C}$   
104 (20D). Light treatments consisted of a daily cycle of 12 hours of light ( $160\text{-}180\ \mu\text{mol}$   
105  $\text{photons m}^{-2}\text{ s}^{-1}$ ) and 12 hours of complete darkness. Dark treatments consisted of 12 hours  
106 of very low light ( $<10\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$ ) and 12 hours of complete darkness. Two extra  
107 microcosms with glass tiles were previously incubated under 20L conditions for 28 days in  
108 order to obtain a mature biofilm for testing sensitivity of oxygen consumption (measured  
109 by the optode technique) to biofilm age (and correspondingly, thickness). Random samples

were selected from each microcosms, and used with the CLSM and spatial optode techniques.

## **2.2 Confocal laser scanning microscopy (CLSM): images on biofilm architecture**

CLSM (Leica TCS-SP5 AOBS CLSM, Leica Microsystems Heidelberg GmbH, Mannheim, Germany) was performed to obtain images that were relevant to assess the spatial organization of biofilms for the four components visualized: algae, cyanobacteria, EPS, and DNA. After day 7, one replicate per treatment was transferred into an individual flask with enough water to allow the analysis in vivo by CLSM.

Autofluorescence of photosynthetic pigments was viewed in the red channel for phycobiliproteins (570–615 nm emissions) using a 561nm laser diode (to detect cyanobacteria) and in the blue channel (670–790 nm emissions) for chlorophylls using a 594 nm Helium-Neon laser (to detect algae). EPS was stained with lectin concanavalin A

(ConA)-Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR) and observed in the green channel (495 to 550 nm emissions) using a 488-nm Argon laser. DNA (as a proxy to visualize bacteria) was stained with Hoechst (Hoechst 33342, Molecular Probes, Invitrogen, H-3570), excited by UV light at 405 nm, and observed in the blue/cyan fluorescence light at 414-458 nm.

For three dimensional analysis, 20 slices were recorded, separated at a fixed distance of 1 micrometer. This is valid for all treatments and for all variables, such that the statistics were computed over samples of the same size (i.e., the same volume was considered for all the treatments and biofilm components), following the same approach used to determine the respiration rates. To decide the depth and number of slices to adopt,

we first measured the maximum depths of the different biofilms in different locations within the biofilm (up, down, left, right). Then, based on this initial inspection, we chose 20  $\mu\text{m}$  to include the maximum volume of biofilm from all biofilm types. Image analyses were performed with the IMARIS code (v. 6.1.0 software, Bitplane, Zürich, Switzerland). Images were taken at different magnification levels. The analysis of the spatial organization of the biofilm structures was performed on the one-week old 10X images that allow the analysis of algae, cyanobacteria and EPS, but not of bacteria which was analyzed only in the 40X images. Most of the results presented in this paper correspond to the 10X images, corresponding to 512x512 pixels, each of size  $0.76 \times 0.76 \mu\text{m}^2$  (representing a subset of the total image equal to a square of side 0.39 mm). The lenses used were PlanApochromatic 40X (NA 1.25, oil) and PlanApochromatic 10X/0.40 CS. The direct comparison of the 10X and 40X images was not possible given that the two set of images were obtained using lenses with different resolution (in the planar direction) and signal elongation (in axial direction). The purpose of using the 40X images, is solely to relate the microbial respiration to the amount of DNA.

### **2.3 Visisens: image acquisition and sensor foil calibration**

The system consisted of a USB-Microscope device (VisiSens; PreSens GmbH, Regensburg, Germany) with an embedded software. The technique is based on the dynamic quenching of a luminescence indicator by oxygen. Using the LEDs incorporated into the USB microscope, the indicator and reference dyes immobilized in the sensor foil (SF-RPSU4, Presens GmbH, Regensburg, Germany) are simultaneously excited with the same blue light source. The red light emitted by the reference dye is not affected by oxygen changes. On the contrary, the green light emitted by the indicator dye is quenched by  $\text{O}_2$ .

Since the emission wavelengths of the red and green lights match the red and green channel sensitivity of a RGB chip, both signals are recorded within a single image. Quantification was obtained by the ratio of the red and green channels of the RGB image applying a calibration function derived from the sensor output. The calibration function was determined from exposure to known oxygen concentration values, which were 0% (obtained covering the sensor foil with sodium sulfite solution) and 100% (obtained exposing the sensor foil to air). We refer the reader to Holst and Grunwald (2001) and Gansert and Blossfeld (2008) for a detailed description of the method.

The oxygen-sensitive foils were cut into 1cm<sup>2</sup> squares and glued with silicone to the inner bottom of 6 well-plates (polystyrene, Nunc). The sensitive side of the foil was in direct contact with the biofilm grown glass tiles. Superficial oxygen concentration distribution was recorded with the Visisens unit detector. Measurements were performed on the one-week biofilms grown at the four conditions of temperature and light described, as

well as in four-week old biofilms grown at 20°C in light conditions. The image accumulated for an area of 1280x1024 pixels (1mm=115pixel). Images were recorded every 30 seconds over 30 minutes (a total of 60 images per treatment). Spatial averaging and hotspot evaluations were performed by using the z-function in the Vivisens software. A schematic overview of the setup can be found in the supplementary material.

## 2.4 Statistical analysis

CLSM images were analyzed for spatial distribution description, based on the 2D projections along the vertical axis of the different color bands for the 10X visualization fields. Intensity values (for each channel) were measured by an *ad-hoc* script in Matlab. The intensity values for each channel (chlorophyll, phycobilin, concanavaline, DNA)



178 varying in a 0-255 scale, were converted to binary sets, after selecting a threshold light  
179 intensity, so that at each pixel the variable  $I(x, y)$ , indicated presence ( $I=1$ ) or no presence  
180 ( $I=0$ ), based on whether the actual measured light intensity at each pixel exceeded the  
181 threshold value. For all channels, the threshold value chosen was set to 10% of the  
182 maximum potential intensity (i.e., corresponding to an intensity value of 25). This threshold  
183 was determined by visual inspection between the resulting binary map and that of the  
184 original images with intensity color plots. The sensitivity of the choice of threshold was  
185 tested in the red channel, using also a value of 50, and then comparing the results of the  
186 statistical analysis. Finally, we extended the work to the green and blue channels, and found  
187 no reason to use a threshold different from 25 (again, from visual inspection of the resulting  
188 binary maps).

189 The resulting binary (indicator) fields were analyzed for the number of non-void pixels, and  
190 then the omnidirectional sampled variograms were obtained (horizontal isotropy in the

191 plots was adopted based on preliminary tests). The variograms were then fitted to standard  
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192 existing models.

193 To account for the heterogeneity of the biofilm samples, we used two stacks of images for  
194 each set of environmental conditions. For each stack, we used a sampling window large  
195 enough to guarantee that the ergodicity of the sample was ensured (i.e. we checked in  
196 preliminary analysis that sampled windows were at least 25 times larger than the largest  
197 integral scale for all components and all treatments). For all treatments and channels, the  
198 ranges obtained in the two stacks were quite similar (see Table 1 in the SI). Note that  
199 ergodicity could not be assessed properly in the 40X images (due to the smaller sampled  
200 domain size). Therefore, the ranges determined for the bacteria may not be representative

of the whole biofilm. However, we included these data, since they were collected at the center of the sample where the respiration rates were measured and, as stated before, the purpose of using the 40X images is solely to relate the microbial respiration to the amount of DNA.

Note that, in our study, the small proportion of non-void pixels close to the substratum and the comparison of the 3D and 2D images indicated that the statistics for the one-week old biofilm were preserved in the 2D projections (see Appendix A). Thus, the 2D projections allowed obtaining the statistics of the aggregates sizes and the planar distance between aggregates. However, the use of 2D projections in older/denser biofilm, may result in artifacts and requires further investigations.

The combination of univariate (histograms) and bivariate statistics (indicator variograms) in the 2d projected images provided simple but very useful information about the way the different components of the biofilm are spatially organized under different conditions of light and temperature.

The rates of oxygen consumption were measured using oxygen concentration values taken every 30 sec in the interval 0 to 30 min for each individual sample. Non-linear regression analyses using Sigmaplot 11.0 (Systatsoftware, Inc CA. USA) were performed in each data set in order to calculate the slope of the oxygen concentration versus time plot, equivalent to the consumption rate.

### **3. Results**

#### **3.1 Geostatistical analysis of confocal images.**

Figure 1 represents the 3D images (at 10X magnification) of the biofilms grown at the four environmental conditions (10L, 20L, 10D, and 20D) and for all four biofilm compartments analyzed in this work (algae, cyanobacteria, EPS, and bacteria). These images revealed the presence of highly structured microbial aggregations, whose sizes and distribution varied depending on both temperature and light conditions. More precisely, the 20L biofilm CLSM image exhibited the largest number of total non-void pixels of all the compartments, with algae, cyanobacteria, and EPS unevenly distributed within the biofilm. On the contrary, the 10D image was characterized by largest amounts of empty pixels.

**Figure 1 goes here**

The amount of area occupied by cyanobacteria, EPS and algae (approximated by the proportion of non-void pixels) ranged from 0.12 for EPS in one of the 10D stack, to 15.4% for algae in the 20L biofilm in the 2d projections, as shown in Figure 1b. The 10D treatment presented always the lowest values for all the three components. In the 10D and 20D, the largest amounts of non-void pixels corresponded to cyanobacteria while in the 10L and 20L, algae were the most abundant component (see Figure 2 and Table 1).

**Figure 2 goes here**

The CLSM images were interpreted by variography of the binary images to determine selected spatial characteristic sizes and statistical parameters of the biofilm compartments analyzed. The variogram associated to a stochastic process is a powerful tool to describe how the variable of interest correlates as a function of distance. The sample variogram is then fitted by one of the standard stationary models. In our analyses, we studied the number of structures required to model the experimental variograms of the 10X images for algae,

cyanobacteria, and EPS. For each one of them, we obtained a fitted range (i.e., the distance beyond which data are not spatially correlated), equivalent to the average size of the microbial aggregates for each component; also, each structure displays a sill, that represents the fraction of the total variance explained.

The statistical analysis for the 3D images revealed that microbes were mostly concentrated in the bottom slices (results not shown). The vertical dimension displayed a slightly larger range than the horizontal one, but the actual estimate of range is very uncertain due to the limited number of slices available. As a consequence, the approach presented in this work based on 2D projections was devised only for thin biofilms, with sizes of hundreds of microns in the x-y directions and only a few micrometers in the z direction, and would not be useful for thick (mature) biofilms. While the statistical descriptors obtained for the 2D and 3D cases were not equal, the overall behavior was similar, and thus the 2D projected data was considered suitable to study the spatial structure of the different biofilm compartments. In any case, an example of the comparison between the 2D and the 3D images for the 10D red channel is included in Appendix A. In the following, we report only the 2D results.

The variables explored for the 2D projections included the three channels observed in the 10X images: cyanobacteria (red - R), EPS (green - G), and algae (blue - B). The resulting sample variograms were then fitted by two superimposed (nested) stationary models with no nugget, providing two sills (their sum corresponds approximately to the total variance of the indicator parameter, indicative of a stationary stochastic process) and two ranges (Table 1). Stationarity implies that the aggregates are placed throughout all the domain, without any spatial trend, or in other words, that aggregates grow following a

similar pattern in different areas. The first variogram model displayed a short range being an indication of the size of the smallest aggregates, and accounted for most of the variability (on the average, it explains 72% of the total variance); the second one showed a larger range, indicating the characteristic distance between aggregates, and it completes the full variance description. In the following, we refer to them as small-scale and large-scale aggregates respectively. The different sample variograms could be fitted by means of exponential models (thus, displaying no continuity at the origin). This is an indication that the shapes of the aggregates are not smooth, but irregular.

**Table 1 goes here**

Overall, the average size of both the small- and large-scale aggregates of biofilms grown at 20°C were larger than those grown at 10°C. In contrast, aggregate sizes associated to light variations (dark vs. light) were just slightly different depending on the channel analyzed. For cyanobacteria (R - channel), the short-range variogram structure (small-scale aggregates) explained 59% to 81% of the corresponding total variance, and provided ranges (i.e. average sizes) of 2-3.9 pixels (the average is equivalent to 2.2  $\mu\text{m}$ ), just slightly larger under 20°C dark conditions (Table 1). The second structure (large-scale aggregates) showed ranges of 10-42  $\mu\text{m}$  (13-55 pixels) and 22-33  $\mu\text{m}$  (29-41.5 pixels) for biofilms incubated under dark and light conditions, respectively. The combination of variogram ranges and percentage of occupied pixels for cyanobacteria indicated that small-scale aggregates are quite similar regardless of channels or environmental conditions; on the other hand, the number of pixels occupied is largest under light conditions, indicating that in such a case there was a larger number of cyanobacteria distributed throughout the domain forming average size aggregates.

For EPS, the small-scale aggregates ranged between 1.6  $\mu\text{m}$  (10D) and 3  $\mu\text{m}$  (20D), describing 53%-78% of the total variance, while for algae the average size was between 2.1  $\mu\text{m}$  (10D) to 2.3  $\mu\text{m}$  (20D and 10L) and accounted for 64%-80% of the total variance. The range of the second structure (large-scale aggregates) was quite variable, being of 4.6-27.8  $\mu\text{m}$  for EPS and 9.1-32.7  $\mu\text{m}$  for algae, with a clear increase with temperature and light. Again, the combination of ranges and non-void pixel percentage, indicates that for the 10D treatment, biofilm occupied just a very reduced area that tended to be localized in very few clusters; on the contrary, for the 20L case, aggregates were distributed all along the area, with a large number of clusters covering the full space.

Bacteria (DNA stain) could only be assessed in the images with a 40X magnification and therefore are treated separately. The results from the variographic analysis are reported in Table 2. In the 10D biofilm the area occupied by bacteria was the lowest of all treatments (see also the inset in Figure 2), and their location displayed no apparent spatial correlation, characteristic of an uncorrelated random function. The remaining three experimental variograms could be studied using second order stationary models. Due to the high-resolution images it was not possible to obtain large ranges (anything above 100 pixels; here 1 pixel = 0.19  $\mu\text{m}$ ). In all three cases, the sampled variogram could be fitted by a single model with a nugget effect indicating a combination of variability at a very low range (uncorrelated random process), representing 60-65% of the total variability, and a correlated process that accounted for the remaining 35-40% of the variance, with ranges of 2.3  $\mu\text{m}$ , 6.5  $\mu\text{m}$  and 7.8  $\mu\text{m}$  for treatments 10L, 20D, and 20L, respectively. This indicates that clustering of bacteria is more sensitive to temperature than to light conditions.

**Table 2 goes here**

### 3. 2 Oxygen respiration patterns

Metabolic processes in biofilms are the result of autotrophic and heterotrophic organisms happening at the  $\mu\text{m}$  to  $\text{mm}$  scale. Oxygen is produced by photosynthetic microorganisms and consumed by aerobic respiration. To study the resulting spatial distribution of oxic/anoxic zones in the biofilms at the micro-scale, we monitored the changes of oxygen concentration on the surface of the 10D, 10L, 20D, 20L and mature biofilms over thirty minutes at a temporal frequency of 30 seconds. Figure 3 shows the spatially averaged oxygen concentration for the one-week-old biofilms grown at 10D, 10L, 20D and 20L and for a (four-week-old) mature biofilm grown at 20° C under light conditions. The mature biofilm had an initial oxygen concentration of 160% (expressed as % of air saturation) due to the high colonization of algae and cyanobacteria. This value was comparable to the initial oxygen concentration for the 20D and 20L biofilms, which ranged 140-160%. Oxygen concentration initial values were lower (60-80%) for 10D and 10L.

The associated oxygen concentration consumption rates were obtained by fitting an exponential decay curve,  $S = a \exp(-bt)$ , on top of the oxygen saturation ( $S$ ) versus time ( $t$ ) data displayed in Figure 3. The values of the coefficients  $a$  (in percentage saturation) and  $b$  (in  $\text{min}^{-1}$ ) are listed in Table 3. The slope of the oxygen decay curve ( $b$ ) increased with temperature, with the largest value measured for the mature biofilm.

**Figure 3 goes here**

**Table 3 goes here**

The real-time images of oxygen concentration at selected times is illustrated in Figure 4 for all 7-day old biofilms, as well as for a 28-day old biofilm grown at 20L (an example

of the whole time series for the 20L biofilm is included in the Supplementary Material, see Video S1). Anaerobic hotspots, defined as zones with disproportionally high reaction rates with respect to the surrounding matrix (McClain et al., 2003) developed under all environmental conditions, but were most intense for the biofilms grown under light.

**Figure 4 goes here**

An example of the fine-scale spatial variability (i.e., heterogeneity) in oxygen decay is presented in Figure 5, corresponding to the one-week 20L biofilm. Five subzones (of 12 mm<sup>2</sup> surface area) were cropped from the original plot. The evolution of the spatially averaged oxygen saturation is plotted, for each subzone, as a function of time. The oxygen decay rates presented a very large variability of the *a* and *b* coefficients, with values ranging from  $b = 0.03 - 0.10 \text{ min}^{-1}$ .

**Figure 5 goes here**

Histograms of the spatial oxygen concentration distribution are presented in Figure 6 for two selected times, 3 minutes and 30 minutes. Figure 6 displays a large variability in space among the oxygen concentration histograms studied. Overall, the largest reduction in oxygen concentration is observed for the mature biofilm. For young biofilms, the largest reduction is observed in the 20L treatment. In almost all the young biofilms the initial oxygen concentration distribution is positively skewed, with a mean ranging 87-95% air saturation, and a median close to 100% air saturation. The only exception is the 10L case that presented a negatively skewed distribution with a low initial value (58% air saturation).

All the 30 minute oxygen concentration histograms were positively skewed, with mean air saturation values ranging between 53% and 32%. Contrarily, the oxygen concentration



histograms of the mature biofilm were very different, with initial distribution mostly symmetrical and an average value of 167% air saturation, that decreased very rapidly to 0.6% at  $t=30$  minutes.

**Figure 6 goes here**

### **3.3 Linking biofilm structure to oxygen respiration rates**

The heterogeneous distribution of oxygen concentration in space and time (highlighted in Figure 4 to Figure 6) can be understood from a combination of statistical and biological considerations. The oxygen concentration maps have a pixel size of about  $8.7\text{ }\mu\text{m}$ , therefore always larger than the range of the smallest biofilm structure determined by the variogram analysis (i.e., characteristic length scale of the small-scale aggregates). On the other hand,  $8.7\text{ }\mu\text{m}$  is smaller in general (comparable in the 10D case) than the ranges obtained for the second structure. This suggests that all pixels in the oxygen concentration maps have some amount of biofilm (non-void pixels) that can produce/consume oxygen. In addition, the variographic analysis performed (at the 10X resolution) indicates only presence/no-presence of biofilm, but does not provide information about activity. Therefore, it is expected that the oxygen dynamics is variable both in space and time. Finally, biofilm respiration rates have been traditionally described by a lognormal model (e.g., del Giorgio and Williams, 2005; Forney and Rothman, 2012), thus it can be expected that the statistical distribution of oxygen concentration values displays a positively skewed, describable by a lognormal distribution, as shown in Figure 6.

Figure 7 explores the potential correlations between the biofilm structure and the respiration rates for the one-week old biofilms analyzed in this study. To this aim, the

panels in Figure 7 displays the values of the respiration rates,  $b$  (see Table 3), *versus* the amount of non-void pixel (i.e., the amount of biofilm for each of its components) and *versus* the corresponding ranges of both the small and large scale aggregates. Additionally, in Figure 7b we show the amount of non-void pixel of DNA *versus* the respiration rates, stressing that these values were obtained with the 40X images. Results show that in all cases there is positive correlation between oxygen decay and the proportion of non-void pixels. This is regardless of the biofilm component analyzed (phycobilins, EPS, or Chl-a).

**Figure 7 goes here**

#### **4. Discussion and conclusions**

The 3D spatial structure of the biofilm analyzed in this study showed the presence of microbial structures tightly associated, whose size and spatial distribution were strongly modulated by different environmental conditions. Previous studies have already shown the effect of light and temperature on biofilm structure by CLSM observations (e.g., Díaz Villanueva et al., 2011). Our work represents a step further in the analysis of biofilm structures, by quantifying the spatial distribution and characteristic scales of the microbial assemblages of algae, EPS, cyanobacteria and bacteria. To this aim, the geostatistical analysis of the CLSM images represents a powerful tool to determine the most significant features of the microbial spatial distribution at the micro-scale.

The 3D information based on 20 slices separated 1 micrometer, indicated that most of the biological structures were located at the bottom slices, impeding to assess the statistical stationarity along the vertical. Therefore, the 2D projection of the 3D data was used to

study the one-week-old biofilms. The sampled variograms of the 2D projection displayed a well-marked sill, characteristic of a stationary variable (no observed spatial trends), and the best fit was obtained by a nested model of two exponential components. The range of the first structure provided the characteristic length of small aggregates of 1.5-3  $\mu\text{m}$ , quite constant regardless environmental conditions. More, the small-scale aggregates were not located following an independent (Poisson-type) distribution, but rather, part of the total variance (an average of 28%) was explained by a second structure with somewhat larger ranges, indicating the presence of middle-size aggregates whose size is highly dependent on temperature and light conditions. Additionally, the lack of continuity at the origin in the variogram models, indicated that the aggregates presented an irregular shape (far from an spherical shape).

In the case of cyanobacteria, the variographic analysis indicated that the largest range is observed for the 20D case, suggesting that smaller aggregates could be observed under light conditions than under dark ones, and in the dark such larger aggregates, are more scattered in space. As expected, the number of pixels occupied by cyanobacteria was larger under light than dark conditions for a given temperature. The differences in ranges between dark/light treatments could be related to the capacity of cyanobacteria to adapt their morphology based on light condition (Iijima et al. 2015). For instance, cyanobacteria have been found to exhibit elongated shapes when grown under low light conditions (de Marsac et al 1993), and in our study this is visible for the 20 °C.

On the contrary, in the case of bacteria, clustering was more sensitive to temperature than to light conditions, suggesting that bacteria clusters included a majority of heterotrophs, whose growth is favored by temperature and non-affected by the light

conditions. For algae, main differences were found on the size of large-scale aggregates, which increased with light and temperature, and could be related to a change in species composition such as greater abundance of filamentous green algae (Pillsbury and Lowe, 1999). The ranges for EPS were quite similar to those of cyanobacteria (around 20% smaller in the latter), as EPS are expected to be placed surrounding the cells. The relative area occupied by EPS under 10L conditions was unexpectedly larger than in 20D and 20L; however, Varin et al. (2012) found that cyanobacteria may produce large amount of EPS in cold environments to enable optimal growth at low temperatures.

The characteristic spatial scales of the aggregates were then compared to the respiration rates measured in the biofilms grown at different conditions of light and temperature to investigate the link between biofilm function and structure. Variations in respiration rates were observed between the one-week (young) and the four-week (mature) biofilms. In addition, respiration rates increased in biofilms grown at 20°C, as compared to those grown at 10°C, and no differences were observed for light conditions. The fact that the 10L biofilm presented similar respiration rates than the 10D, although in the former a larger area was occupied by microbes, may suggest that microorganisms of biofilm grown under dark conditions are more efficient (higher respiration activity per cell) than the ones of biofilms grown under light conditions. The latter may contain a largest presence of inactive cells, as observed for natural grown biofilms (Romaní et al., 2004).

The temporal evolution of the 2D oxygen concentration distribution highlighted the development of micro-environments with steep oxygen gradients in all the samples analyzed. In statistical terms, the distribution of oxygen concentration was positively skewed and resembled lognormal distributions both after 3 minutes and 30 minutes of the

respiration monitoring period. Note that the average size of the pixels in the oxygen maps (8.7  $\mu\text{m}$ ) was comparable to the spatial scale of the large aggregate, being about 11 times greater than the size of the pixel in the confocal images (0.76  $\mu\text{m}$ ). Thus, most of the pixels in the oxygen maps contained some non-void cells of biofilms, and respiration occurred in mostly all pixels. That is, for all environmental conditions there was a fast transition during the first minutes of respiration, with a significant reduction for all pixels and a positively skewed distribution, which has often been found to adequately describe the distribution of the microbial populations (e.g., Hirano et al., 1982; Hosoda et al., 2011). Overall, we found that higher values of respiration rates were associated to larger amount of non-void pixels of bacteria, suggesting that microbial respiration was responsible for the oxygen decay depicted at the fine (mm) scale. We conclude that: (i) using geostatistical analyses is a valuable tool to quantify the size and spatial pattern of microbial aggregates, and (ii) the combination of optodes and CLMS is a promising way to relate the oxidative metabolic activity to the biofilm architecture at the micrometric scale.

## **Appendix A: 3D vs. 2D characterization**

This appendix aims to compare the statistical analysis for the 3D and 2D projection of the 10D condition for cyanobacteria (red channel). A total of 512x512x18 pixels were analyzed, with only 0.03% being considered positive, i.e., occupied by biofilm. Positive pixels were mostly concentrated in the bottom slices. For the 3D case, the sample variogram in the horizontal direction was fitted by the superposition of two models, both spherical. The first one accounted for 90% of the total variance and had a range of 2 pixels (=1.5  $\mu\text{m}$ ). The range of the second one was of 15 pixels (about 11  $\mu\text{m}$ ). Similar to the 3D

field, the sample variogram of the 2D projection was fitted by the superposition of a short- and a medium-range structures, both exponential. The first one accounts for 91% of the total variance and has a range of 3.6 pixels (=2.6  $\mu\text{m}$ ). The second one accounts for the remaining 9% variance, with a 10 pixel (=7.3  $\mu\text{m}$ ) range. The total amount of occupied pixels is 0.033%.

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## REFERENCES

- Battin TJ, Sengschmitt D (1999) Linking sediment biofilms, hydrodynamics, and river bed clogging: Evidence from a large river. *Microb. Ecol.* 37:185–196.
- Battin TJ, Kaplan LA, Newbold JD, Hansen CM (2003). Contributions of microbial biofilms to ecosystem processes in stream mesocosms. *Nature* 426(6965):439-442.
- Battin, T. J., Besemer, K., Bengtsson, M. M., Romani, A. M., & Packmann, A. I. (2016). The ecology and biogeochemistry of stream biofilms. *Nature Reviews Microbiology*, 14(4), 251-263.

490 Bozorg A, Gates ID, Sen A (2012) Real time monitoring of biofilm development under  
 491 flow conditions in porous media. *Biofouling* 28(9):937-951.

492 Costerton, J. W., Geesey, G. G. & Cheng, K. J. How bacteria stick. *Sci. Am.* 238, 86–95  
 493 (1978).

494 de Marsac, N. T., & Houmard, J. (1993). Adaptation of cyanobacteria to environmental  
 495 stimuli: new steps towards molecular mechanisms. *FEMS microbiology reviews*, 10(1-  
 496 2), 119-189. Del Giorgio, P.A., le B Williams, PJ (2005) Respiration in aquatic  
 497 ecosystems, Oxford University Press.

498 Díaz Villanueva V, Font J, Schwartz T, Romaní AM (2011) Biofilm formation at warming  
 499 temperature: acceleration of microbial colonization and microbial interactive effects.  
 500 *Biofouling*, 27(1):59-71.

501 Fenchel T, Finlay B (2008). Oxygen and the spatial structure of microbial  
 502 communities. *Biological Reviews*, 83(4):553-569.

503 Forney DC, Rothman DH (2012) Inverse method for estimating respiration rates from  
 504 decay time series, *Biogeosciences*, 9, 3601-3612, doi:10.5194/bg-9-3601-2012.

505 Gette-Bouvarot M, Mermillod-Blondin F, Angulo-Jaramillo R, Delolme C, Lemoine D,  
 506 Lassabatere L, Loizeau S, Volatier L (2014) Coupling hydraulic and biological  
 507 measurements highlights the key influence of algal biofilm on infiltration basin  
 508 performance. *Ecohydrology* 7(3), 950-964, doi: 10.1002/eco.1421.

509 Hirano SS, Nordheim EV, Arny DC, Upper CD (1982) Lognormal Distribution of  
 510 Epiphytic Bacterial Populations on Leaf Surfaces. *Appl Environ Microbiol.* 44(3), 695–  
 511 700.

512 Hosoda K, Matsuura T, Suzuki H, Yomo T (2011) Origin of lognormal-like distributions  
 513 with a common width in a growth and division process. *Physical Review E* 83, 031118.

514 Hödl I, Hödl J, Wörman A, Singer G, Besemer K, Battin TJ (2011) Voronoi tessellation  
 515 captures very early clustering of single primary cells as induced by interactions in  
 516 nascent biofilms. *PLoS One* 6, e26368.

517 Iijima, H., Shirai, T., Okamoto, M., Kondo, A., Hirai, M. Y., & Osanai, T. (2015). Changes  
 518 in primary metabolism under light and dark conditions in response to overproduction  
 519 of a response regulator RpaA in the unicellular cyanobacterium *Synechocystis* sp. PCC  
 520 6803. *Frontiers in microbiology*, 6.

521 Kim J-W, Choi H, Pachepsky YA (2010) Biofilm morphology as related to the porous  
 522 media clogging. *Water Research* 44(4): 1193-1201.

523 Kühl M, Rickelt LF, Thar R (2007) Combined imaging of bacteria and oxygen in  
 524 biofilms. *Applied and Environmental Microbiology* 73(19):6289-6295.

525 Kühl M, Polerecky L (2008) Functional and structural imaging of phototrophic microbial  
 526 communities and symbioses. *Aquatic Microbial Ecology* 53(1):99.

527 McClain, M. E., Boyer, E. W., Dent, C. L., Gergel, S. E., Grimm, N. B., Groffman, P. M.,  
 528 ... & McDowell, W. H. (2003). Biogeochemical hot spots and hot moments at the  
 529 interface of terrestrial and aquatic ecosystems. *Ecosystems*, 6(4), 301-312.



530 Neu TR, Lawrence JR (2016) Laser Microscopy for the Study of Biofilms: Issues and  
 531 Options. In: Romaní AM, Guasch H, Balaguer MD, Aquatic Biofilms, Ecology, Water  
 532 Quality and Wastewater treatment, Caister Academic Press, Norfolk, UK, pp 29-45.

533 Nguyen T, Roddick FA, Fan L (2012) Biofouling of water treatment membranes: A review  
 534 of the underlying causes, monitoring techniques and control measures. *Membranes* 2,  
 535 804-840; doi:10.3390/membranes2040804.

536 Peter H, Ylla I, Gudas C, Romaní AM, Sabater S, Tranvik LJ (2011) Multi-functionality  
 537 and diversity in bacterial biofilms. *PLoS One*, 6(8), e23225.

538 Proia L, Romaní AM, Sabater S (2012). Nutrients and light effects on stream biofilms: a  
 539 combined assessment with CLSM, structural and functional parameters.  
 540 *Hydrobiologia* 695(1):281-291.

541 Pillsbury R.W., Lowe R.L., (1999). The response of benthic algae to manipulation of light  
 542 in four acidic lakes in northern Michigan. *Hydrobiologia* 394: 69 – 81.

543 Romaní AM, Guasch H, Muñoz I, Ruana J, Vilalta E, Schwartz T, Emtiazi F, Sabater S  
 544 (2004) Biofilm structure and function and possible implications for riverine DOC  
 545 dynamics. *Microb. Ecol.* 47:316–328.

546 Rubol, S., Freixa, A., Carles-Brangari, A., Fernàndez-Garcia, D., Romaní, A. M., &  
 547 Sanchez-Vila, X. (2014). Connecting bacterial colonization to physical and  
 548 biochemical changes in a sand box infiltration experiment. *Journal of Hydrology*, 517,  
 549 317-327.

- Rubol, S., Dutta, T., & Rocchini, D. (2016). 2D visualization captures the local heterogeneity of oxidative metabolism across soils from diverse land-use. *Science of The Total Environment*, 572, 713-723.
- Salta, M., Wharton, J.A., Blache, Y., Stokes, K.R., and Briand, J.F. (2013). Marine biofilms on artificial surfaces: structure and dynamics. *Env. Microb.* 15(11), 2879-2893.
- Singer G, Besemer K, Schmitt-Kopplin P, Hödl I, Battin TJ (2010) Physical heterogeneity increases biofilm resource use and its molecular diversity in stream mesocosms. *Plos One*, 5(4), e9988.
- Staal M, Borisov SM, Rickelt LF, Klimant I, Kühl M (2011a) Ultrabright planar optodes for luminescence life-time based microscopic imaging of O<sub>2</sub> dynamics in biofilms. *Journal of Microbiological Methods* 85(1):67-74.
- Staal M, Prest EI, Vrouwenvelder JS, Rickelt LF, Kühl M (2011b) A simple optode based method for imaging O<sub>2</sub> distribution and dynamics in tap water biofilms. *Water Research* 45(16):5027-5037.
- Varin, T., Lovejoy, C., Jungblut, A. D., Vincent, W. F., & Corbeil, J. (2012). Metagenomic analysis of stress genes in microbial mat communities from Antarctica and the High Arctic. *Applied and environmental microbiology*, 78(2), 549-559.

## List of Tables

**Table 1.** Statistical (variographic) analysis of the CLSM 2D projections from the 10X magnification images for cyanobacteria (red channel; R), EPS (green channel; G) and algae

(blue channel; B). The table includes the proportion of non-void pixels ( $p$ ), and the variance ( $p(1-p)$  in a binary field), as well as the parameters representing a two-nested statistical structure, each range indicative of the size of aggregates and each sill their relative contribution to the total variance. Values are means for the two analyzed stacks.

**Table 2.** Statistical analysis of the different models indicating the fraction of pixels occupied by bacteria (DNA stain, grey channel), and the parameters corresponding to the variographic study. Analysis was performed on the 40X magnification images. In this case, only the range of the smallest aggregate could be assessed.

**Table 3.** Coefficients  $a$  and  $b$  (with standard deviations in parenthesis) representing the parameters of exponential regression for mature (28 days old) and young (7-day) biofilms growing at different conditions of light and temperature. The regression coefficients  $R^2$  are also reported.

## List of Figures

**Figure 1.** Top: 3D CLSM images (corresponding to 10X magnification) of biofilm grown over 7 days for the four combinations of environmental conditions of light and temperature: 10D, 20D, 10L and 20L. The different biofilm compartments are shown in red (phycobilins autofluorescence, cyanobacteria), green (concanavalin stain, EPS), blue (chlorophyll autofluorescence, algae), and light grey (DNA stain, bacteria). Bottom: the corresponding two-dimensional projections.

**Figure 2.** Percentage of non-void pixels of cyanobacteria, algae and EPS corresponding to the 2D projections of the 3D CLSM 10X magnification images for the one-week-old biofilms. In the case of bacteria (shown in the inset), 40X images were used for the analysis. Values are the average from the two stacks analyzed per biofilm.

**Figure 3.** Spatially averaged oxygen values (expressed as % air saturation) for the one-week-old biofilms grown under different conditions of light and temperature and for a mature (four-week-old) biofilm grown at 20L conditions. Bars represent the standard deviation. The specific rates calculated for each decay curve are summarized in Table 3.

**Figure 4.** Maps of oxygen concentrations at selected times (initial time 0 and then every 10 min), highlighting the formation of anoxic hotspots and the high variability of oxygen concentrations in space and time for the one-week-old biofilms (four treatments), and the mature biofilm (28-day-old).

**Figure 5.** An example of the highly heterogeneous oxygen concentration distribution and oxygen concentration decay rates for the 20L biofilm. The oxygen map corresponds to  $t=30$  min. The area is divided into 5 subzones, and the evolution of oxygen saturation as a function of time is shown for the each subzones.

**Figure 6.** Histograms of the oxygen distribution at  $t=3$  min (left column) and  $t=30$  min (middle column) for young biofilms and treatments 10D, 10L, 20D, 20L and for a mature biofilm treatment (28-day-old) grown at 20L. The vertical light blue lines indicate 100% air

618 saturation Right: Binary field for oxygen content at  $t=30$  min (in black pixels with oxygen  
619 content of less than 40% air saturation).

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621 **Figure 7.** Relationship between respiration rate (coefficient  $b$  in Table 3) and different  
622 statistics related to the CLSM (proportion of non-void pixels and ranges of the nested  
623 variograms).

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